4.5-kb DNA insertion. While this carrying capacity may prevent the AAV from delivering large genes, it is amply suited for delivering the antisense constructs of the present invention.

AAV is also a good choice of delivery vehicles due to its safety. There is a relatively complicated rescue mechanism: not only wild type adenovirus but also AAV genes are required to mobilize rAAV. Likewise, AAV is not pathogenic and not associated with any disease. The removal of viral coding sequences minimizes immune reactions to viral gene expression, and therefore, rAAV does not evoke an inflammatory response.

D. Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention for the delivery of oligonucleotide or polynucleotide sequences to a host cell. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Coupar *et al*., 1988), lentiviruses, polio viruses and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* (1991) introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

E. Non-viral vectors

In order to effect expression of the oligonucleotide or polynucleotide sequences of the present invention, the expression construct must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states. As described above, one preferred mechanism for delivery is *via* viral infection where the expression construct is encapsulated in an infectious viral particle.

Once the expression construct has been delivered into the cell the nucleic acid encoding the desired oligonucleotide or polynucleotide sequences may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the construct may be stably integrated into the genome of the cell. This integration may be in the specific location and orientation *via* homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In certain embodiments of the invention, the expression construct comprising one or more oligonucleotide or polynucleotide sequences may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty & Reshef (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun

and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular gene may be delivered *via* this method and still be incorporated by the present invention.

VII. POLYPEPTIDE COMPOSITIONS

The present invention, in other aspects, provides polypeptide compositions. Generally, a polypeptide of the invention will be an isolated polypeptide (or an epitope, variant, or active fragment thereof) derived from a mammalian species. Preferably, the polypeptide is encoded by a polynucleotide sequence disclosed herein or a sequence which hybridizes under moderately stringent conditions to a polynucleotide sequence disclosed herein. Alternatively, the polypeptide may be defined as a polypeptide which comprises a contiguous amino acid sequence from an amino acid sequence disclosed herein, or which polypeptide comprises an entire amino acid sequence disclosed herein.

Immunogenic portions may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. As used herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.*, they react with the protein in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well known techniques. An immunogenic portion of a *Mycobacterium* sp. protein is a portion that reacts with such antisera and/or T-cells at a level that is not substantially less than the reactivity of the full length polypeptide (*e.g.*, in an ELISA and/or T-cell reactivity assay). Such immunogenic portions may react within such assays at a level that is similar to or greater than the reactivity of the full length polypeptide. Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988). For example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example, ^{125}I-labeled Protein A.

Polypeptides may be prepared using any of a variety of well known techniques. Recombinant polypeptides encoded by DNA sequences as described above may be readily prepared from the DNA sequences using any of a variety of expression vectors known to those of ordinary skill in the art. Expression may be achieved in any appropriate

host cell that has been transformed or transfected with an expression vector containing a DNA molecule that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast, and higher eukaryotic cells, such as mammalian cells and plant cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO. Supernatants from suitable host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide.

Polypeptides of the invention, immunogenic fragments thereof, and other variants having less than about 100 amino acids, and generally less than about 50 amino acids, may also be generated by synthetic means, using techniques well known to those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc.* 85:2149-2146 (1963). Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

Within certain specific embodiments, a polypeptide may be a fusion protein that comprises multiple polypeptides as described herein, or that comprises at least one polypeptide as described herein and an unrelated sequence, such as a known tumor protein. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the protein or to enable the protein to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the protein.

Fusion proteins may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion protein is expressed as a recombinant protein, allowing the production of increased levels, relative to a non-fused protein, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the

DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion protein that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*, *Gene* 40:39-46 (1985); Murphy *et al.*, *Proc. Natl. Acad. Sci. USA* 83:8258-8262 (1986); U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

Fusion proteins are also provided. Such proteins comprise a polypeptide as described herein together with an unrelated immunogenic protein. Preferably the immunogenic protein is capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see, e.g.*, Stoute *et al.*, *New Engl. J. Med.* 336:86-91 (1997)).

Within preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium *Haemophilus influenza* B (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.*, the first N-terminal 100-110 amino acids), and a protein D derivative may

be lipidated. Within certain preferred embodiments, the first 109 residues of a lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemagliutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene* 43:265-292 (1986)). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology* 10:795-798 (1992)). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion protein. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

In general, polypeptides (including fusion proteins) and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

VIII. T CELLS

Immunotherapeutic compositions may also, or alternatively, comprise T cells specific for a *Mycobacterium* antigen. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood, or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system, such as the Isolex™ System, available from

Nexell Therapeutics, Inc. (Irvine, CA; *see also* U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human mammals, cell lines or cultures.

T cells may be stimulated with a polypeptide of the invention, polynucleotide encoding such a polypeptide, and/or an antigen presenting cell (APC) that expresses such a polypeptide. Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide. Preferably, the polypeptide or polynucleotide is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

T cells are considered to be specific for a polypeptide of the invention if the T cells specifically proliferate, secrete cytokines or kill target cells coated with the polypeptide or expressing a gene encoding the polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen *et al., Cancer Res.* 54:1065-1070 (1994)). Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Contact with a polypeptide of the invention (100 ng/ml - 100 μg/ml, preferably 200 ng/ml-25 μg/ml) for 3 - 7 days should result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN-γ) is indicative of T cell activation (*see* Coligan *et al., Current Protocols in Immunology*, vol. 1 (1998)). T cells that have been activated in response to a polypeptide, polynucleotide or polypeptide-expressing APC may be $CD4^+$ and/or $CD8^+$. Protein-specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient, a related donor or an unrelated donor, and are administered to the patient following stimulation and expansion.

For therapeutic purposes, $CD4^+$ or $CD8^+$ T cells that proliferate in response to a polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example,

the T cells can be re-exposed to a polypeptide, or a short peptide corresponding to an immunogenic portion of such a polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a r polypeptide. Alternatively, one or more T cells that proliferate in the presence of ar protein can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution.

IX. PHARMACEUTICAL COMPOSITIONS

In additional embodiments, the present invention concerns formulation of one or more of the polynucleotide, polypeptide, T-cell and/or antibody compositions disclosed herein in pharmaceutically-acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy.

It will also be understood that, if desired, the nucleic acid segment, RNA, DNA or PNA compositions that express a polypeptide as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, other proteins or polypeptides or various pharmaceutically-active agents. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA or DNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

A. Oral Delivery

In certain applications, the pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; Hwang *et al.*, 1998; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. A syrup of elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as one

containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

B. Injectable Delivery

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion (*see, e.g., Remington's Pharmaceutical Sciences*, 15th Edition, pp. 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The compositions disclosed herein may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

C. Nasal Delivery

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described *e.g.*, in U. S. Patent 5,756,353 and U. S. Patent 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U. S. Patent 5,780,045 (specifically incorporated herein by reference in its entirety).

D. Liposome-, Nanocapsule-, and Microparticle-Mediated Delivery

In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the compositions of the present invention into suitable host cells. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically-acceptable formulations of the nucleic acids or constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon & Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath & Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta & Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller & Baltimore, 1984), transcription factors and allosteric effectors (Nicolau & Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985a; 1985b; Coune, 1988; Sculler *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori & Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They

are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1988), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Alternatively, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to

intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

X. VACCINES

In certain preferred embodiments of the present invention, vaccines are provided. The vaccines will generally comprise one or more pharmaceutical compositions, such as those discussed above, in combination with an immunostimulant. An immunostimulant may be any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. Examples of immunostimulants include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into which the compound is incorporated; *see, e.g.*, Fullerton, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example, Powell & Newman, eds., *Vaccine Design* (the subunit and adjuvant approach) (1995). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other tumor antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine.

Illustrative vaccines may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems* 15:143-198 (1998), and references cited therein. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication

competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch *et al.*, *Proc. Natl. Acad. Sci. USA* 86:317-321 (1989); Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86-103 (1989); Flexner *et al.*, *Vaccine* 8:17-21 (1990); U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques* 6:616-627 (1988); Rosenfeld *et al.*, *Science* 252:431-434 (1991); Kolls *et al.*, *Proc. Natl. Acad. Sci. USA* 91:215-219 (1994); Kass-Eisler *et al.*, *Proc. Natl. Acad. Sci. USA* 90:11498-11502 (1993); Guzman *et al.*, *Circulation* 88:2838-2848 (1993); and Guzman *et al.*, *Cir. Res.* 73:1202-1207 (1993). Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer *et al.*, *Science* 259:1745-1749 (1993) and reviewed by Cohen, *Science* 259:1691-1692 (1993). The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells. It will be apparent that a vaccine may comprise both a polynucleotide and a polypeptide component. Such vaccines may provide for an enhanced immune response.

It will be apparent that a vaccine may contain pharmaceutically acceptable salts of the polynucleotides and polypeptides provided herein. Such salts may be prepared from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.*, salts of primary, secondary and tertiary amines and basic amino acids) and inorganic bases (*e.g.*, sodium, potassium, lithium, ammonium, calcium and magnesium salts).

While any suitable carrier known to those of ordinary skill in the art may be employed in the vaccine compositions of this invention, the type of carrier will vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344 and 5,942,252. One may also employ a carrier comprising the particulate-protein complexes described in U.S. Patent

No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

Such compositions may also comprise buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate. Compounds may also be encapsulated within liposomes using well known technology.

Any of a variety of immunostimulants may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium* species or *Mycobacterium* derived proteins. For example, delipidated, deglycolipidated *M. vaccae* ("pVac") can be used. In another embodiment, BCG is used. In addition, the vaccine can be administered to a subject previously exposed to BCG. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 and derivatives thereof (SmithKline Beecham, Philadelphia, PA); CWS, TDM, Leif, aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Within the vaccines provided herein, the adjuvant composition is preferably designed to induce an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines

will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann & Coffman, *Ann. Rev. Immunol.* 7:145-173 (1989).

Preferred adjuvants for use in eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPL adjuvants are available from Corixa Corporation (Seattle, WA; *see* US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.*, *Science* 273:352 (1996). Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins . Other preferred formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A, β-escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamelar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol® to increase viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

In one preferred embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL® adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. Another particularly

preferred adjuvant formulation employing QS21, 3D-MPL® adjuvant and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 as disclosed in WO 00/09159. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*. SBAS-2, AS2', AS2,'' SBAS-4, or SBAS6, available from SmithKline Beecham, Rixensart, Belgium), Detox (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, the disclosures of which are incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants such as those described in WO 99/52549A1.

Other preferred adjuvants include adjuvant molecules of the general formula (I): $HO(CH_2CH_2O)_n$-A-R,

wherein, n is 1-50, A is a bond or -C(O)-, R is C_{1-50} alkyl or Phenyl C_{1-50} alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein *n* is between 1 and 50, preferably 4-24, most preferably 9; the *R* component is C_{1-50}, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether, polyoxyethylene-9-steoryl ether, polyoxyethylene-8-steoryl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12^{th} edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immune response enhancer and a suitable carrier or excipient. The compositions described herein may be administered as part of a sustained

release formulation (*i.e.*, a formulation such as a capsule, sponge or gel (composed of polysaccharides, for example) that effects a slow release of compound following administration). Such formulations may generally be prepared using well known technology (*see, e.g.*, Coombes *et al.*, *Vaccine* 14:1429-1438 (1996)) and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane.

Carriers for use within such formulations are biocompatible, and may also be biodegradable; preferably the formulation provides a relatively constant level of active component release. Such carriers include microparticles of poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.*, a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see, e.g.*, U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune response that targets tumor cells. Delivery vehicles include antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau& Steinman, *Nature* 392:245-251 (1998)) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman & Levy, *Ann. Rev. Med.* 50:507-529 (1999)). In general, dendritic cells may be

identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel *et al.*, *Nature Med.* 4:594-600 (1998)).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNFα to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNFα, CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.*, CD54 and CD11) and costimulatory molecules (*e.g.*, CD40, CD80, CD86 and 4-1BB).

APCs may generally be transfected with a polynucleotide encoding a protein (or portion or other variant thereof) such that the polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally

be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi *et al.*, *Immunology and Cell Biology* 75:456-460 (1997). Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (*e.g.*, vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (*e.g.*, a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

XI. DIAGNOSTIC KITS

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a protein of the invention.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

XII. EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1: Recombinant Fusion Proteins of *M. tuberculosis* Antigens Exhibit Increased Serological Sensitivity

A. Materials and Methods

1. Construction of vectors encoding fusion proteins: TbF14

TbF14 is a fusion protein of the amino acid sequence encoding the MTb81 antigen fused to the amino acid sequence encoding the Mo2 antigen. A sequence encoding Mo2 was PCR amplified with the following primers: PDM-294 (T_m 64°C) CGTAATCACGTGCAGAAGTACGGCGGATC (SEQ ID NO:14)and PDM-295 (T_m 63°C) CCGACTAGAATTCACTATTGACAGGCCCATC (SEQ ID NO:15).

DNA amplification was performed using 10 µl 10X Pfu buffer, 1 µl 10 mM dNTPs, 2 µl each of the PCR primers at 10 µM concentration, 83 µl water, 1.5 µl Pfu DNA polymerase (Stratagene, La Jolla, CA) and 50 ng DNA template. For Mo2 antigen, denaturation at 96°C was performed for 2 min; followed by 40 cycles of 96°C for 20 sec, 63°C for 15 sec and 72°C for 2.5 min; and finally by 72°C for 5 min.

A sequence encoding MTb81 was PCR amplified with the following primers: PDM-268 (T_m 66°C) CTAAGTAGTACTGATCGCGTGTCGGTGGGC (SEQ ID NO:16) and PDM-296 (T_m 64°C) CATCGATAGGCCTGGCCGCATCGTCACC (SEQ ID NO:17). The amplification reaction was performed using the same mix as above, as follows: denaturation at 96°C for 2 min; followed by 40 cycles of 96°C for 20 sec, 65°C for 15 sec, 72°C for 5 min; and finally by 72°C for 5 min.

The Mo2 PCR product was digested with Eco72I (Stratagene, La Jolla CA) and EcoRI (NEB, Beverly, MA). The MT681 PCR product was digested with FseI and StuI (NEB, Beverly, MA). These two products were then cloned into an expression plasmid (a modified pET28 vector) with a hexahistidine in frame, in a three way ligation that was digested with FseI and EcoRI. The sequences was confirmed, then the expression plasmid was transformed into the BL21pLysE *E. coli* strain (Novagen, Madison, WI) for expression of the recombinant protein.

2. Construction of vectors encoding fusion proteins: TbF15

TbF15 is a fusion of antigens Ra3, 38 kD (with an N-terminal cysteine), 38-1, and FL TbH4 from *Mycobacterium tuberculosis*, as was prepared a follows. TbF15 was made using the fusion constructs TbF6 and TbF10.

TbF6 was made as follows (see PCT/US99/03268 and PCT/US99/03265). First, the FL (full-length) TbH4 coding region was PCR amplified with the following primers:PDM-157 CTAGTTAGTACTCAGTCGCAGACCGTG (SEQ ID NO:18) (T_m 61°C) and PDM-160 GCAGTGACGAATTCACTTCGACTCC (SEQ ID NO:19) (T_m 59°C), using the following conditions: 10 µl 10X Pfu buffer, 1µl 10 mM dNTPs, 2 µl 10 µM each oligo, 82 µl sterile water, 1.5 µl Accuzyme (ISC, Kaysville, UT), 200 ng *Mycobacterium tuberculosis* genomic DNA. Denaturation at 96°C was performed for 2 minutes; followed by 40 cycles of 96°C for 20 seconds, 61°C 15 seconds, and 72°C 5 minutes; and finally by 72°C 10 minutes.

The PCR product was digested with ScaI and EcoRI and cloned into pET28Ra3/38kD/38-1A, described below, which was digested with DraI and EcoRI.

pET28Ra3/38kD/38-1A was made by inserting a DraI site at the end of 38-1 before the stop codon using the following conditions. The 38-1 coding region was PCR amplified with the following primers: PDM-69 GGATCCAGCGCTGAGATGAAGACCGATGCCGCT (SEQ ID NO:19) (T_m 68°C) and PDM-83 GGATATCTGCAGAATTCAGGTTTAAAGCCCATTTGCGA (SEQ ID NO:20) (T_m 64°C), using the following conditions: 10 µl 10X Pfu buffer, 1 µl 10 mM dNTPs, 2µl 10 µM each oligo, 82µl sterile water, 1.5 µl Accuzyme (ISC, Kaysville, UT), 50 ng plasmid DNA. Denaturation at 96°C was performed for 2 minutes; followed by forty cycles of 96°C for 20 seconds, 66°C for 15 seconds and 72°C for 1 minute 10 seconds; and finally 72°C 4 minutes.

The 38-1 PCR product was digested with Eco47III and EcoRI and cloned into the pT7ΔL2Ra3/38kD construct (described in WO/9816646 and WO/9816645) which was digested with EcoRI and Eco47III. The correct construct was confirmed through sequence analysis. The Ra3/38kD/38-1A coding region was then subcloned into pET28 His (a modified pET28 vector) at the NdeI and EcoRI sites. The correct construct (called TbF6) was confirmed through sequence analysis.

Fusion construct TbF10, which replaces the N-terminal cysteine of 38 kD, was made as follows. To replace the cysteine residue at the N-terminus, the 38kD-38-1 coding region from the TbF fusion (described in WO/9816646 and WO/9816645) was amplified using the following primers: PDM-192 TGTGGCTCGAAACCACCGAGCGGTTC (SEQ ID NO:21) (T_m 64°C) and PDM-60 GAGAGAATTCTCAGAAGCCCATTTGCGAGGACA (SEQ ID NO:22) (T_m 64°C), using the following conditions: 10 µl 10X Pfu buffer, 1 µl 10 mM dNTPs, 2 µl 10 µM each oligo, 83 µl sterile water, 1.5 µl Pfu DNA polymerase (Stratagene, La Jolla, CA), and 50 ng plasmid TbF DNA. The amplification reaction was performed as follows: 96°C for 2 minutes; followed by 40 cycles of 96°C for 20 seconds, 64°C 15 seconds, and 72°C 4 minutes; and finally 72°C 4 minutes. Digest the PCR product with Eco RI and clone into pT7ΔL2Ra3 which has been digested with Stu I and Eco RI. Digest the resulting construct with Nde I and EcoRI and clone into pET28 at those sites. The resulting clone (called TbF10) will be TBF + a cysteine at the 5' end of the 38kD coding region. Transform into BL21 and HMS 174 with pLys S.

The pET28TbF6 (TbF6, described above) construct was digested with StuI (NEB, Beverly, MA) and EcoRI, which released a 1.76 kb insert containing the very back portion of the 38 kD/38-1/FL TbH4 fusion region. This insert was gel purified. The pET28TbF10 construct (TbF10, described above) was digested with the same enzymes and the vector backbone, consisting of 6.45 kb containing the his-tag, the Ra3 coding region and most of the Δ38kD coding region. This insert was gel purified. The insert and vector were ligated and transformed. The correct construct, called TbF15, was confirmed through sequence analysis, then transformed into the BL21 pLysS *E. coli* strain (Novagen, Madison WI). This fusion protein contained the original Cys at the amino terminus of the 38 kD protein.

B. Expression of fusion proteins

1. Expression of fusion proteins

The recombinant proteins were expressed in *E. coli* with six histidine residues at the amino-terminal portion using the pET plasmid vector and a T7 RNA polymerase expression system (Novagen, Madison, WI). *E. coli* strain BL21 (DE3) pLysE (Novagen) was used for high level expression. The recombinant (His-Tag) fusion proteins were purified from the soluble supernatant or the insoluble inclusion body of 1 L of IPTG induced batch cultures by affinity chromatography using the one step QIAexpress Ni-NTA Agarose matrix (QIAGEN, Chatsworth, CA) in the presence of 8M urea.

Briefly, 20 ml of an overnight saturated culture of BL21 containing the pET construct was added into 1 L of 2x YT media containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol, grown at 37°C with shaking. The bacterial cultures were induced with 1 mM IPTG at an OD 560 of 0.3 and grown for an additional 3 h (OD ≈ 1.3 to 1.9). Cells were harvested from 1 L batch cultures by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate, pH 8.0; 10 mM Tris-HCl, pH 8.0) containing 2 mM PMSF and 20 μg/ml leupeptin plus one complete protease inhibitor tablet (Boehringer Mannheim) per 25 ml. *E. coli* was lysed by freeze-thaw followed by brief sonication, then spun at 12 k rpm for 30 min to pellet the inclusion bodies.

The inclusion bodies were washed three times in 1% CHAPS in 10 mM Tris-HCl (pH 8.0). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea or 8M urea was added directly into the soluble supernatant. Recombinant fusion proteins with His-Tag residues were batch bound to Ni-NTA agarose resin (5 ml resin per 1 L inductions) by rocking at room temperature for 1 h and the complex passed over a column. The flow through was passed twice over the same column and the column washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCl, pH 6.3) also containing 8 M urea. Bound protein was eluted with 30 ml of 150 mM imidazole in wash buffer and 5 ml fractions collected. Fractions containing each recombinant fusion protein were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0) bound one more time to the Ni-NTA matrix, eluted and dialyzed in 10 mM Tris-HCl (pH 7.8). The yield of recombinant protein varies from 25-150 mg per liter of induced bacterial culture with greater than 98% purity. Recombinant proteins were assayed for endotoxin contamination using the *Limulus* assay (BioWhittaker) and were shown to contain < 100 E.U./mg.

2. Serological assays

ELISA assays were performed with TbF15 using methods known to those of skill in the art, with 200 ng/well of antigen. ELISA assays are performed with TbF14 using methods known to those of skill in the art, with 200 ng/well of antigen.

3. Results

The TbF15 fusion protein containing TbRa3, 38kD (with N terminal cysteine), Tb38-1, and full length (FL) TbH4 as described above was used as the solid phase antigen in ELISA. The ELISA protocol is as described above. The fusion recombinant was coated at 200 ng/well. A panel of sera were chosen from a group of TB patients that had previously been shown by ELISA to be positive or borderline positive with these antigens. Such a panel enabled the direct comparison of the fusions with and without the cysteine residue in the 38 kD component. The data are outlined in Figure 5. A total of 23 TB sera were studied and of these 20/23 were detected by TbF6 versus 22/23 for TbF15. Improvements in reactivity were seen in the low reactive samples when TbF15 was used.

One of skill in the art will appreciate that the order of the individual antigens within each fusion protein may be changed and that comparable activity would be expected provided that each of the epitopes is still functionally available. In addition, truncated forms of the proteins containing active epitopes may be used in the construction of fusion proteins.

Example 2: Cloning, construction, and expression of HTCC#1 full-length, overlapping halves, and deletions as fusion constructs

HTCC#1 (aka MTb40) was cloned by direct T cell expression screening using a T cell line derived from a healthy PPD positive donor to directly screen an *E. coli* based MTb expression library.

A. Construction and screening of the plasmid expression library

Genomic DNA from *M. tuberculosis* Erdman strain was randomly sheared to an average size of 2 kb and blunt ended with Klenow polymerase, before EcoRI adaptors were added. The insert was subsequently ligated into the 1 screen phage vector and packaged *in vitro* using the PhageMaker extract (Novagen). The phage library (Erd 1 screen) was amplified and a portion was converted into a plasmid expression library. Conversion from phage to plasmid (phagemid) library was performed as follows: the Erd 1 Screen phage library was converted into a plasmid library by autosubcloning using the *E. coli* host strain BM25.8 as suggested by the manufacturer (Novagen). Plasmid DNA was purified from BM25.8 cultures containing the pSCREEN recombinants and used to transform competent

cells of the expressing host strain BL21(DE3)pLysS. Transformed cells were aliquoted into 96 well micro titer plates with each well containing a pool size of ~50 colonies. Replica plates of the 96 well plasmid library format were induced with IPTG to allow recombinant protein expression. Following induction, the plates were centrifuged to pellet the *E. coli* and the bacterial pellet was resuspended in 200 µl of 1X PBS.

Autologous dendritic cells were subsequently fed with the *E. coli*, washed and exposed to specific T cell lines in the presence of antibiotics to inhibit the bacterial growth. T cell recognition was detected by proliferation and/or production of IFN-γ. Wells that score positive were then broken down using the same protocol until a single clone could be detected. The gene was then sequenced, sub-cloned, expressed and the recombinant protein evaluated.

B. Expression in *E. coli* of the full-length and overlapping constructs of HTCC#1

One of the identified positive wells was further broken down until a single reactive clone (HTCC#1) was identified. Sequencing of the DNA insert followed by search of the Genebank database revealed a 100% identity to sequences within the *M. tuberculosis* locus MTCY7H7B (gene identification MTCY07H7B.06) located on region B of the cosmid clone SCY07H7. The entire open reading frame is ~1,200 bp long and codes for a 40 kDa (392 amino acids) protein (Fig. 1; HTCC#1 FL). Oligonucleotide PCR primers [5'(5'-CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC *ATG AGC AGA GCG TTC ATC ATC*-3') and 3' (5'-CAT GGA ATT CGC CGT TAG ACG ACG TTT CGT A-3')] were designed to amplify the full-length sequence of HTCC#1 from genomic DNA of the virulent Erdman strain.

The 5' oligonucleotide contained an *Nde I* restriction site preceding the ATG initiation codon (underlined) followed by nucleotide sequences encoding six histidines (bold) and sequences derived from the gene (italic). The resultant PCR products was digested with NdeI and EcoRI and subcloned into the pET17b vector similarly digested with NdeI and EcoRI. Ligation products were initially transformed into *E. coli* XL1-Blue competent cells (Stratagene, La Jolla, CA) and were subsequently transformed into *E. coli* BL-21 (pLysiE) host cells (Novagen, Madison, WI) for expression.

C. Expression of the full length and overlapping constructs of HTCC#1

Several attempts to express the full-length sequence of HTCC#1 in *E. coli* failed either because no transformants could be obtained or because the *E. coli* host cells were

lysed following IPTG induction. HTCC#1 is 392 amino acids long and has 3 trans-membrane (TM) domains which are presumably responsible for the lysing of the *E. coli* culture following IPTG induction.

Thus expression of HTCC#1 was initially attempted by constructing two overlapping fragments coding for the amino (residues 1-223; Fig. 2a) and carboxy (residues 184-392; Fig. 2b) halves.

The N-terminal (residues 1-223) fragment containing the first of the 3 putative transmembrane domains killed (lysed) the host cells, while the C-terminal (residues 184-392) half expressed at high levels in the same host cell. Thus the two trans-membrane domains located in the C-terminal half do not appear to be toxic.

The N-terminal fragment, comprising amino acid residues 1-128 (devoid of the transmembrane domain), was therefore engineered for expression in the same pET17b vector system (Fig. 2c). This construct expressed quite well and there was no toxicity associated with the expressing *E. coli* host.

D. Expression in *E. coli* of the full-length HTCC#1 as an TbRa12 fusion construct

Because of problems associated with the expression of full length HTCC#1, we evaluated the utility of an TbRa12 fusion construct for the generation of a fusion protein that would allow for the stable expression of recombinant HTCC#1.

pETl7b vector (Novagen) was modified to include TbRa12, a 14 kDa C-terminal fragment of the serine protease antigen MTB32A of *Mycobacterium tuberculosis* (Skeiky *et al.*). For use as an expression vector, the 3' stop codon of the TbRa12 was substituted with an in frame EcoRI site and the N-terminal end was engineered so as to code for six His-tag residues immediately following the initiator Met. This would facilitate a simple one step purification protocol of TbRa12 recombinant proteins by affinity chromatography over Ni-NTA matrix.

Specifically, the C-terminal fragment of antigen MTB32A was amplified by standard PCR methods using the oligonucleotide primers 5'(CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC *ACG GCC GCG TCC GAT AAC TTC* and 3' (5'-CTA ATC GAA TTC *GGC CGG GGG TCC CTC GGC CAA*). The 450 bp product was digested with NdeI and EcoRI and cloned into the pET17b expression vector similarly digested with the same enzymes.

Recombinant HTCC#1 was engineered for expression as a fusion protein with TbRa12 by designing oligonucleotide primers to specifically amplify the full length form.

The 5' oligonucleotide contained a thrombin recognition site. The resulting PCR amplified product was digested with EcoRI and subcloned into the EcoRI site of the pET-TbRa12 vector. Following transformation into the *E. coli* host strain (XL1-blue; Stratagene), clones containing the correct size insert were submitted for sequencing in order to identify those that are in frame with the TbRa12 fusion. Subsequently, the DNA of interest (Fig. 3) was transformed into the BL-21 (pLysE) bacterial host and the fusion protein was expressed following induction of the culture with IPTG.

E. Expression in *E. coli* of HTCC#1 with deletions of the trans-membrane domain(s)

Given the prediction that the 3 predicted trans-membrane (TM) domains are responsible for lysing the *E. coli* host following IPTG induction, recombinant constructs lacking the TM domains were engineered for expression in *E. coli*.

1. Recombinant HTCC#1 with deletion of the first TM (ΔTM-1). A deletion construct lacking the first trans-membrane domain (amino acid residues 150-160) was engineered for expression *E. coli* (Fig. 4a). This construct expressed reasonably well and enough (low mg quantities) was purified for *in vitro* studies. This recombinant antigen was comparable in *in vitro* assays to that of the full-length Ra-12-fusion construct.

T-cell epitope mapping of HTCC#1. Because of the generally low level of expression using the ΔTM-1 construct, the design of the final form of HTCC#1 for expression in *E. coli* was based on epitope mapping. The T-cell epitope was mapped using 30 overlapping peptides (Fig. 4b) on PBMC read out (on four PPD+ donors). The data revealed that peptides 8 through 16 (amino acid residues 92-215) were not immunogenic (Fig. 4c).

2. Recombinant HTCC#1 with deletion of all of the TM domains (ΔTM-2): A deletion construct of HTCC#1 lacking residues 101 to 203 with a predicted molecular weight of 30.4 kDa was engineered for expression in *E. coli*. The full length HTCC#1 is 40 kDa. There was no toxicity associated with this new deletion construct and the expression level was higher than that of the ΔTM-1 construct (Fig. 4d).

F. Fusion constructs of HTCC#1 and TbH9:

Fig. 5 shows a sequence of HTCC#1 (184-392)-TbH9-HTCC#1 (1-129)

Fig. 6 shows a sequence of HTCC#1 (1-149)-TbH9-HTCC#1 (161-392)

Fig. 7 shows a sequence of HTCC#1 (184-392)-TbH9-HTCC#1 (1-200)

One of skill in the art will appreciate that the order of the individual antigens within each fusion protein may be changed and that comparable activity would be expected provided that each of the epitopes is still functionally available. In addition, truncated forms of the proteins containing active epitopes may be used in the construction of fusion proteins.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising an MTb81 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and an Mo2 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

2. The composition of claim 1, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

3. The composition of claim 2, wherein the fusion polypeptide has the amino acid sequence of TbF14.

4. A pharmaceutical composition comprising a TbRa3 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a 38kD antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a Tb38-1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a FL TbH4 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

5. The composition of claim 4, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

6. The composition of claim 5, wherein the fusion polypeptide has the amino acid sequence of TbF15.

7. A pharmaceutical composition comprising an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

8. The composition of claim 7, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

9. The composition of claim 7, comprising a full-length HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, and a full-length TbH9 antigen from a *Mycobacterium* species of the tuberculosis complex.

10. The composition of claim 9, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

11. The composition of claim 10, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(FL)-TbH9(FL).

12. The composition of claim 7, comprising a polypeptide comprising amino acids 184-392 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a polypeptide comprising amino acids 1-129 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex.

13. The composition of claim 12, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

14. The composition of claim 13, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(184-392)/TbH9/HTCC#1(1-129).

15. A pharmaceutical composition comprising a TbRa12 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

16. The composition of claim 15, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

17. The composition of claim 16, wherein the fusion polypeptide has the amino acid sequence of TbRa12-HTCC#1.

18. A pharmaceutical composition comprising at least two heterologous antigens from a *Mycobacterium* species of the tuberculosis complex or an immunogenic fragment thereof, wherein the antigen or immungenic fragment thereof is selected from the group consisting of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14

(Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI (Mtb9.9A, also known as MTI-A), ESAT-6, α-crystalline, and 85 complex.

19. The composition of claim 18, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

20. The composition of claim 1, 4, 7, 15, or 18, wherein the antigens are covalently linked via a chemical linker.

21. The composition of claim 20, wherein the chemical linker is an amino acid linker.

22. The composition of claim 1, 4, 7, 15, or 18, further comprising at least one additional antigen from a *Mycobacterium* species of the tuberculosis complex, wherein the antigen is selected from the group consisting of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14 (Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI (Mtb9.9A, also known as MTI-A), ESAT-6, α-crystalline, and 85 complex, or an immunogenic fragment thereof.

23. The composition of claim 1, 4, 7, 15, or 18, further comprising an adjuvant.

24. The composition of claim 23, wherein the adjuvant comprises QS21 and MPL.

25. The composition of claim 23, wherein the adjuvant is selected from the group consisting of AS2, ENHANZYN, MPL, QS21, CWS, TDM, AGP, CPG, Leif, saponin, and saponin mimetics.

26. The composition of claim 1, 4, 7, 15, or 18, further comprising BCG.

27. The composition of claim 1, 4, 7, 15, or 18, further comprising an NS1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

28. The composition of claim 1, 4, 7, 15, or 18, wherein the *Mycobacterium* species is *Mycobacterium tuberculosis*.

29. An expression cassette comprising a nucleic acid encoding an MTb81 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding an Mo2 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

30. The expression cassette of claim 29, wherein the nucleic acid encodes a fusion polypeptide comprising an MTb81 antigen or an immunogenic fragment thereof and a nucleic acid encoding an Mo2 antigen or an immunogenic fragment thereof.

31. The expression cassette of claim 30, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbF14.

32. The expression cassette of claim 31, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbF14.

33. An expression cassette comprising a nucleic acid encoding a TbRa3 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a nucleic acid encoding a 38kD antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a nucleic acid encoding a Tb38-1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a FL TbH4 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

34. The expression cassette of claim 33, wherein the nucleic acid encodes a fusion polypeptide comprising a TbRa3 antigen or an immunogenic fragment thereof, a 38kD antigen or an immunogenic fragment thereof, a Tb38-1 antigen or an immunogenic fragment thereof, and a nucleic acid encoding a FL TbH4 antigen or an immunogenic fragment thereof.

35. The expression cassette of claim 34, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbF15.

36. The expression cassette of claim 35, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbF15.

37. An expression cassette comprising a nucleic acid encoding an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

38. The expression cassette of claim 37, comprising a nucleic acid encoding a full-length HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a full-length TbH9 antigen from a *Mycobacterium* species of the tuberculosis complex.

39. The expression cassette of claim 37, comprising a nucleic acid encoding a polypeptide comprising amino acids 184-392 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, a nucleic acid encoding a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a polypeptide comprising amino acids 1-129 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex.

40. The expression cassette of claim 37, wherein the nucleic acid encodes a fusion polypeptide comprising an HTCC#1 antigen or an immunogenic fragment thereof, and a TbH9 antigen or an immunogenic fragment thereof.

41. The expression cassette of claim 38, wherein the nucleic acid encodes a fusion polypeptide comprising a full-length HTCC#1 antigen, and a full-length TbH9 antigen.

42. The expression cassette of claim 39, wherein the nucleic acid encodes a fusion polypeptide comprising amino acids 184-392 of an HTCC#1, a TbH9 antigen or an immunogenic fragment thereof, and amino acids 1-129 of an HTCC#1 antigen.

43. The expression cassette of claim 41, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of HTCC#1(FL)-TbH9(FL).

44. The expression cassette of claim 43, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding HTCC#1(FL)-TbH9(FL).

45. The expression cassette of claim 42, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of HTCC#1(184-392)/TbH9/HTCC#1(1-129).

46. The expression cassette of claim 45, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding HTCC#1(184-392)/TbH9/HTCC#1(1-129).

47. An expression cassette comprising a nucleic acid encoding a TbRa12 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

48. The expression cassette of claim 47, wherein the nucleic acid encodes a fusion polypeptide comprising an Ra12 antigen or an immunogenic fragment thereof, and an HTCC#1 antigen or an immunogenic fragment thereof.

49. The expression cassette of claim 48, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbRa12-HTCC#1.

50. The expression cassette of claim 49, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbRa12-HTCC#1.

51. An expression cassette comprising a nucleic acid encoding at least two heterologous antigens from a *Mycobacterium* species of the tuberculosis complex or an immunogenic fragment thereof, wherein the antigen or immunogenic fragment thereof is selected from the group consisting of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14 (Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI (Mtb9.9A, also known as MTI-A), ESAT-6, α-crystallinc, and 85 complex.

52. The expression cassette of claim 51, wherein the nucleic acid encodes a fusion polypeptide.

53. The expression cassette of claim 29, 33, 37, 47 or 51, further comprising a nucleic acid encoding at least one additional antigen from a *Mycobacterium* species of the tuberculosis complex, wherein the antigen is selected from the group consisting

of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14 (Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI, ESAT-6, α-crystalline, and 85 complex, or an immunogenic fragment thereof.

54. The expression cassette of claim 29, 33, 37, 47 or 51, further comprising a nucleic acid encoding an NS1 antigen or an antigenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

55. The expression cassette of claim 29, 33, 37, 47 or 51, wherein the *Mycobacterium* species is *Mycobacterium tuberculosis*.

56. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of a pharmaceutical composition comprising an MTb81 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and an Mo2 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

57. The method of claim 56, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

58. The method of claim 57, wherein the fusion polypeptide has the amino acid sequence of TbF14.

59. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of a pharmaceutical composition comprising a TbRa3 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a 38kD antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a Tb38-1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a FL TbH4 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

60. The method of claim 59, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

61. The method of claim 60, wherein the fusion polypeptide has the amino acid sequence of TbF15.

62. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of a pharmaceutical composition comprising an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

63. The method of claim 62, wherein the pharmaceutical composition comprises a full-length HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, and a full-length TbH9 antigen from a *Mycobacterium* species of the tuberculosis complex.

64. The method of claim 63, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

65. The method of claim 64, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(FL)-TbH9(FL).

66. The method of claim 62, wherein the pharmaceutical composition comprises a polypeptide comprising amino acids 184-392 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a polypeptide comprising amino acids 1-129 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex.

67. The method of claim 66, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

68. The method of claim 67, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(184-392)/TbH9/HTCC#1(1-129).

69. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of a pharmaceutical composition comprising a TbRa12 antigen or an immunogenic fragment

thereof from a *Mycobacterium* species of the tuberculosis complex, and an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

70. The method of claim 69, wherein the antigens are covalently linked, thereby forming a fusion polypeptide.

71. The method of claim 70, wherein the fusion polypeptide has the amino acid sequence of TbRa12-HTCC#1.

72. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of a pharmaceutical composition comprising at least two heterologous antigens from a *Mycobacterium* species of the tuberculosis complex or an immunogenic fragment thereof, wherein the antigen or immunogenic fragment thereof is selected from the group consisting of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14 (Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI (Mtb9.9A, also known as MTI-A), ESAT-6, α-crystalline, and 85 complex.

73. The method of claim 72, wherein the antigens are covalently linked, thereby forming a fusion protein.

74. The method of claim 56, 59, 62, 69, or 72, wherein the mammal has been immunized with BCG.

75. The method of claim 56, 59, 62, 69, or 72, wherein the mammal is a human.

76. The method of claim 56, 59, 62, 69, or 72, wherein the composition is administered prophylactically.

77. The method of claim 56, 59, 62, 69, or 72, wherein the pharmaceutical composition further comprises an adjuvant.

78. The method of claim 77, wherein the adjuvant comprises QS21 and MPL.

79. The method of claim 77, wherein the adjuvant is selected from the group consisting of AS2, ENHANZYN, MPL, QS21, CWS, TDM, AGP, CPG, Leif, saponin, and saponin mimetics.

80. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of an expression cassette comprising a nucleic acid encoding an MTb81 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding an Mo2 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

81. The method of claim 80, wherein the nucleic acid encodes a fusion polypeptide comprising an MTb81 antigen or an immunogenic fragment thereof, and an Mo2 antigen or an immunogenic fragment thereof.

82. The method of claim 81, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbF14.

83. The method of claim 82, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbF14.

84. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of an expression cassette comprising a nucleic acid encoding a TbRa3 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a nucleic acid encoding a 38kD antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a nucleic acid encoding a Tb38-1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a FL TbH4 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

85. The method of claim 84, wherein the nucleic acid encodes a fusion polypeptide comprising a TbRa3 antigen or an immunogenic fragment thereof, a 38kD antigen or an immunogenic fragment thereof, a Tb38-1 antigen or an immunogenic fragment thereof, and a FL TbH4 antigen or an immunogenic fragment thereof.

86. The method of claim 85, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbF15.

87. The method of claim 86, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbF15.

88. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of an expression cassette comprising a nucleic acid encoding an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

89. The method of claim 88, wherein the nucleic acid encodes a fusion polypeptide comprising an HTCC#1 antigen or an immunogenic fragment thereof, and a TbH9 antigen or an immunogenic fragment thereof.

90. The method of claim 89, wherein the nucleic acid encodes a fusion polypeptide comprising a full-length HTCC#1 antigen or an immunogenic fragment thereof, and a full-length TbH9 antigen or an immunogenic fragment thereof.

91. The method of claim 90, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of HTCC#1(FL)-TbH9(FL).

92. The method of claim 91, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding HTCC#1(FL)-TbH9(FL).

93. The method of claim 89, wherein the nucleic acid encodes a fusion polypeptide comprising a polypeptide comprising amino acids 184-392 of an HTCC#1 antigen, a TbH9 antigen or an immunogenic fragment thereof, and a polypeptide comprising amino acids 1-129 of an HTCC#1 antigen.

94. The method of claim 93, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of HTCC#1(184-392)/TbH9/HTCC#1(1-129).

95. The method of claim 93, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding HTCC#1(184-392)/TbH9/HTCC#1(1-129).

96. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of an expression cassette comprising a nucleic acid encoding a TbRa12 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a nucleic acid encoding an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

97. The method of claim 96, wherein the nucleic acid encodes a fusion polypeptide comprising a TbRa12 antigen or an immunogenic fragment thereof, and an HTCC#1 antigen or an immunogenic fragment thereof.

98. The method of claim 97, wherein the nucleic acid encodes a fusion polypeptide having the amino acid sequence of TbRa12-HTCC#1.

99. The method of claim 98, wherein the nucleic acid has the nucleotide sequence of the nucleic acid encoding TbRa12-HTCC#1.

100. A method for eliciting an immune response in a mammal, the method comprising the step of administering to the mammal an immunologically effective amount of an expression cassette comprising a nucleic acid encoding at least two heterologous antigens from a *Mycobacterium* species of the tuberculosis complex or an immunogenic fragment thereof, wherein the antigen or immungenic fragment thereof is selected from the group consisting of MTb81, Mo2, TbRa3, 38kD, Tb38-1 (MTb11), FL TbH4, HTCC#1 (Mtb40), TbH9, MTCC#2 (Mtb41), DPEP, DPPD, TbRa35, TbRa12, MTb59, MTb82, Erd14 (Mtb16), FL TbRa35 (Mtb32A), DPV (Mtb8.4), MSL (Mtb9.8), MTI (Mtb9.9A, also known as MTI-A), ESAT-6, α-crystallins, and 85 complex.

101. The method of claim 100, wherein the nucleic acid encodes a fusion polypeptide.

102. The method of claim 80, 84, 88, 96, or 100, wherein the mammal has been immunized with BCG.

103. The method of claim 80, 84, 88, 96, or 100, wherein the mammal is a human.

104. The method of claim 80, 84, 88, 96, or 100, wherein the composition is administered prophylactically.

105. A fusion protein comprising an MTb81 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and an Mo2 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

106. The protein of claim 105, wherein the fusion polypeptide has the amino acid sequence of TbF14.

107. A fusion protein comprising a TbRa3 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a 38kD antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, a Tb38-1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a FL TbH4 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

108. The protein of claim 107, wherein the fusion polypeptide has the amino acid sequence of TbF15.

109. A fusion protein comprising an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

110. The protein of claim 109, comprising a full-length HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, and a full-length TbH9 antigen from a *Mycobacterium* species of the tuberculosis complex.

111. The protein of claim 110, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(FL)-TbH9(FL).

112. The protein of claim 109, comprising a polypeptide comprising amino acids 184-392 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex, a TbH9 antigen or an immunogenic fragment thereof from a *Mycobacterium*

species of the tuberculosis complex, and a polypeptide comprising amino acids 1-129 of an HTCC#1 antigen from a *Mycobacterium* species of the tuberculosis complex.

113. The protein of claim 112, wherein the fusion polypeptide has the amino acid sequence of HTCC#1(184-392)/TbH9/HTCC#1(1-129).

114. A fusion protein comprising a TbRa12 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex, and an HTCC#1 antigen or an immunogenic fragment thereof from a *Mycobacterium* species of the tuberculosis complex.

115. The protein of claim 114, wherein the fusion polypeptide has the amino acid sequence of TbRa12-HTCC#1.

Figure 1: Nucleotide Sequence of TbF14
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FEATURES	Location/Qualifiers
misc_feature	5072..5095
	/note="His tag coding region"
misc_feature	5096..7315
	/note="MtB81 coding region"
misc_feature	7316..8594
	/note="Mo2 coding region"

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GACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGG
AACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTA
TTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTAC
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Figure 1: Nucleotide Sequence of TbF14
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Figure 1: Nucleotide Sequence of TbF14
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GTCGTCGCCGACCTGACCCCGCAGAACCAAGCTCTGTTGAACGCCCGCGACGAGCTGCAGGCGCAG
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Figure 1: Nucleotide Sequence of TbF14
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GAAGACCCCATCCTGACCGGAGTCGCGCACGACCGCAGCGAGGCCAAGGTGACCATCGTCGGGCTG
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Figure 2: Nucleotide sequence of TbF15
Sheet 1 of 4

FEATURES	Location/Qualifiers
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misc_feature	5096..5293 /note="Ra3 coding region"
misc_feature	5294..6346 /note="38kD coding region"
misc_feature	6347..6643 /note="38-1 coding region"
misc_feature	6644..8023 /note="FL TbH4 coding region"

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